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Dendrogram analysis of multidrug resistant and their relation to some virulence encoding gene of clinical *Pseudomonas aeruginosa*

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Abstract

A total of (60) clinical isolates of gram negative bacteria primary identified as *Pseudomonas aeruginosa* were obtained from different teaching hospitals in Baghdad, the isolates confirmed as *P. aeruginosa* by PCR which was performed by housekeeping gene *(rpsl* gene) these isolates were tested for the sensitivity to 12 antibiotics using disc diffusion method. The ability of the isolates to produce biofilm were tested using Congo red plate method and microtiter plates these measured by optical densities values these values are statistical analyzed by LSD and according to this analysis, the results showed that the statistically difference between the isolates also TCP method is a more quantitative and reliable method for the detection of biofilm. Polymerase chain reaction (PCR) technique was used to screened 5 virulence factor (*exoU, exoS, PilB, PlcH, proteaseIV*) with percentage (53.3%, 26.6%, 21.6%, 0% and 46.6%) positive isolates respectively and these isolates contain either exoU or exoS and according to this have been classified into cytotoxic and invasive. Dendrogram analysis for antibiotics show low discriminatory ability of antibiogram typing because the large number antibiotypes (11 real clone, 15unique pattern), this indicated the reduced ability of the antibiogram to distinguish between *P. aeruginosa* strains.

Keyword: Antibiogram, Dendrogram, Virulence factor.

Introduction

Pseudomonas aeruginosa is often characterized as an opportunistic bacterium which denotes that it rarely causes infection in healthy humans but may do so following disruption of physical barriers and in patients with certain underlying illnesses (Goven, 2007). Outside the hospital setting, skin infections, especially after skin burns and external otitis in frequent swimmers are the most common clinical manifestations (Todar, 2008). Correct diagnosis and treatment of *P. aeruginosa* is severely hampered by the absence of a reliable identification system. A wide range of methods are described for *P. aeruginosa* identification, including conventional methods (e.g. morphology and biochemical tests), indirect molecular strategies and direct molecular strategies involving PCR and sequencing analysis.

The importance of *P. aeruginosa* as an opportunistic pathogen relies in its ability to activate useful phenotypes under environmental stress and to persist in adverse conditions such as the presence of antibiotic or antiseptic substances.

The success of *P. aeruginosa* in diverse environments is attributed to its impressive arsenal of virulence factors, which include multiple cell-associated factors, i.e., alginate (an exopoly-saccharide), lipo-polysaccharide, flagella and pili and secreted virulence factors, including toxins, elastases, protease, phospholipase, as well as small molecules that include phenazines, rhamnolipid and cyanide (Goven, 2007).

The formation of biofilms contributes to the high resistance of *P. aeruginosa* to antibiotics making the treatment of biofilm infections more difficult. In addition, bacteria in biofilm were demonstrated to show elevated resistance to the host immune system clearance (Costerton *et al*., 2003) Factors which explain the high antimicrobial resistance of biofilms include decreased diffusion of antibiotics through the biofilm matrix , decreased oxygen and nutrient , decreased growth rates and metabolism (Momba *et al*., 2000). The major objectives of this study were, To identify and characterize *P. aeruginosa* strains by phenotypic and genotypic methods, to test the antimicrobial susceptibility of *P. aeruginosa* clinical isolates to antibiotics using disk diffusion , To study the relatedness of the strains through dendrogram analysis, to inspect the presence of a relationship between genotypic profiles and antibiotic resistance.

Material and Methods

Sixty gram negative bacterial isolates (primarily diagnosed as *P. aeruginosa*) were obtained from different teaching hospitals in Baghdad during the period from September 2013 to December 2013. They were from various sources. Then were processed as standard protocol. Non-lactose fermenting colonies on MacConkey's agar (MA) were processed and identified as *Pseudomonas aeruginosa* by standard biochemical tests (Atlas *et al*., 1997) and confirmatory by api20 kit.

A housekeeping gene (*rpsL* gene) was used for genotypic detecting by PCR process. Specific primers were used, and were prepared according to the manufacture company information by adding deionized distilled water to obtain stock solution equal to 100pmol/µM. From the previous stock, 10µl was diluted by adding 90 µl distilled water to get a secondary stock concentration equal to 10pmol. Template DNA was prepared as described by Ruppé *et al.* (2009). Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 1ml distilled water and boiled in a water bath, for 10 min. After centrifugation at 10000 rpm for 5min, the suspension was taken as a template. PCR mixture for this gene composed from 12.5µl of GoTaq®Green Master Mix, template DNA 5µl, forward & reverse primers (1.5µl for each), and 4.5 of Deionized Nuclease–Free water was added to PCR mixture to get final volume of 25µl. PCR mixture without template DNA was used as a negative control. PCR was run under the following conditions : primary denaturation step at 95°C for 5min, 30 repeated cycles start with denaturation step at 94°C for 30sec, annealing at 57°C for 30sec, and 1min at 72°C as extension step followed by final extension step at 72°C for 7 min.

Detection of susceptibility to antibacterial agents: Susceptibility of all the isolates to different antibiotics was determined by the disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI). The antibiotic discs used in this study were Amoxicillin/clavulanic acid (30µg) , Tetracycline (10µg), Cefoxitin (30µg), Ceftazidime (10µg), Ceftriaxone (30µg), Ciprofloxacin (10µg), Norfloxacin (10µg), Cefepime (30µg), Chloramphenicol (30µg), Pipracilin (30µg), Tobramycin (30µg), Azithromycin (10µg) . Each antibiotic concentration was applied on the surface of Muller-Hinton agar plates inoculated with *P. aeruginosa* isolates and incubated at 37°C for 24hrs.

Haemolysin production: Plate hemolysis test was done for the detection of β-haemolysin produced by *P. aeruginosa.* The bacteria were inoculated onto 5% human blood agar and incubated over night at 37°C.

Congo red test: Mathur *et al*. (2006) have described method for screening of biofilm formation. Plates were inoculated and incubated aerobically for 24-48hrs. at 37°C. Positive result was indicated by black colonies with a dry crystalline consistency. Weak slime producers usually remained pink, though occasional darkening at the centers of colonies was observed. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated an indeterminate result.

Preparation of bacterial DNA: The DNA to be amplified was extracted from whole organisms by boiling method. The bacteria were harvested from 1.5ml of an overnight Luria-Bertani broth culture, suspended in sterile distilled water, and incubated at 95°C for 10min. Following centrifugation , the supernatant was stored at -20°C as a template DNA stock .

PCR amplification procedure: Detection of virulence genes was performed by amplifying the genes by PCR. The primers sequences were previously reported by and obtained from Alpha DNA company (USA). Descriptions and sequences of the PCR primers used in this study are displayed in Table 1. Amplification was performed in a thermal cycler (Eppendorf, Germany), for *pilB, ExoU*, *ProteaseIV* the reactions mixtures included an initial denaturation at 94°C for 5min consisted of 35 cycles of 94°C for 30sec., specific annealing temperature 60ºC for 30sec. and 72°C for 5min 30sec. and a final extension at 72°C for 10min For *PlcH* the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94°C for 30sec., specific annealing temperature 69◦C for 30sec. and 72ºC for 30sec. and a final extension at 72°C for 10min. for *ExoS* gene the reactions mixtures included an initial denaturation at 94°C for 5 min consisted of 35 cycles of 94ºC for 30sec., specific annealing temperature 65◦C for 30se. and 72°C for 5min in a Thermal Cycler. The detection PCR products was performed on 0.8 to 1% agarose gels by electrophoresis and visualized under UV light.

Results and Discussion

In this study, sixty isolates of *P. aeruginosa* were isolated from different hospitals in Bagdad The source of these isolates were as follows: 22 isolates collected from burn patients, 18 isolates from wounds infections, 9 isolates from sputum taken from patients suffering from respiratory tract infection, 6 isolates from blood, 2 isolates from urinary tract infections (UTI), and the last 3 isolates from ear swab.

Table (1): Sequence of PCR primer and molecular size of PCR product

Gene		Primer (oligonucleotide 5'-3')	Product (bp)	Reference	Origin
rpsI	F	GCAAGCGCATGGTCGACAAGA	201	Xavier *et al*. (2010)	Alpha DNA Co. (Canada)
	R	CGCTGTGCTCTTGCAGGTTGTGA			
ExoU	F	GGG AAT ACT TTC CGG GAA GTT	428	Mitov (2010)	Alpha DNA Co. (Canada)
	R	CGA TCT CGC TGC TAA TGT GTT			
ExoS	F	CTT GAA GGG ACT CGA CAA GG	504	Mitov *et al.* (2010)	Alpha DNA Co. (Canada)
	R	TTC AGG TCC GCG TAG TGA AT			
Protease IV	F	TAT TTC GCC CGA CTC CCT GTA	752	Smith *et al.* (2006)	Alpha DNA Co. (Canada)
	R	AAT AGA CGC CGC TGA AAT C			
PlcH	F	GAA GCC ATG GGC TAC TTC AA	307	Holban *et al.* (2013)	Alpha DNA Co. (Canada)
	R	AGA GTG ACG AGG AGC GGT AG			
PilB	F	ATG AAC GAC AGC ATC CAA CT	826	Mitov *et al.* (2010)	Alpha DNA Co. (Canada)
	R	GGG TGT TGA CGC GAA AGT CGA T			

Table (2): Prevalence *P. aeroginosa* of in clinical specimens

Type of specimen	No. of isolates	*P. aeroginosa* isolates, no. (%)[a]	*Gender,* no. (%)[a]		Dwelling-place, no. (%)[a]	
			Male	Female	Urban	Rural
Burn	22	36.66	15	21.6	31.6	5
Wound	18	30	26.6	3.3	18.3	11.3
Sputum	9	15	6.6	8.3	5	10
Blood	6	10	10	-	8.3	1.6
Ear swab	3	5	5	-	5	-
Urine	2	3.3	1.6	1.6	3.3	-
Total	60	100	64.8	34.8	71.5	27.9

Where ([a]) is the percentage of the number of isolates with respect to the total number of isolates.

Microscopic examination of *P. aeruginosa* showed negative gram reaction, very small rods occur as single bacteria or in pairs. For other biochemical tests, *P. aeruginosa* showed a positive result for oxidase, catalase, while urease test was negative. Final Identification for the isolate have been done at two level: The first was by using conventional method (api 20E) that characterized as the typical easy and rapid one .The second step have been performed by housekeeping gene (rpsL)using polymerase chain reaction technique (PCR) all the (60) isolates gave positive result in both of the previous two steps. Salman *et al*. (2013) pointed to the beneficial use of housekeeping gene in species detection. Moreover Caltoir *et al*. (2000) suggested that PCR is the technique that offers a fast (<1.5h) tool with high sensitivity and specificity for the detection of *P. aeruginosa* as compared to conventional methods.

The resistance to different antibiotics as represented by the diameter in mm. All the isolated shows resistance to more than one antimicrobial. in the present study *P. aeruginosa* isolates were highly resistance against tetracycline 100%, Ceftazidime 100%, Cefoxitin 100%, Ceftriaxone 85%, Cefepime 83.3%, Amoxicillin/Clavulanic acid 81.6%, Chloramphenicol 78.3%, Moderate resistance against, Pipracillin 58.3% and low resistance against, Ciprofloxacin 28.3% Norfloxacin 31.6% Azithromycin 31.6% and Tobramycin 33.3%.

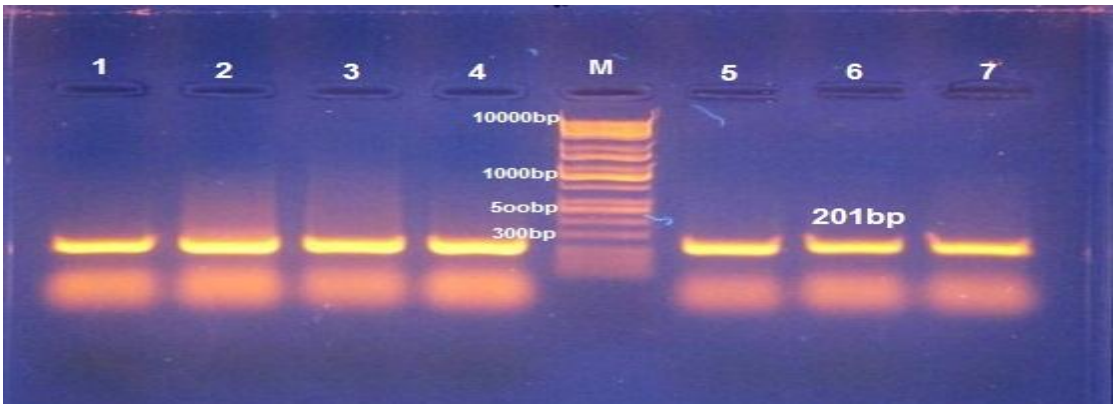

Figure (1): Agarose gel electrophoresis (1% agarose, 7 V/cm2 for 60min) of rpsl gene (201bp amplicon) lane M 100bp DNA ladder, lanes 1-7 represent of bands.

As it is shown in Table (3), all isolates differentiated into 7 pattern according to multiple resistant pattern shows by locally isolates. The highest rate of multidrug resistance was observed with Pattern 1, 2 and 3 in which this isolate was able to resist (10→12)antibiotics. While the lowest multidrug resistance was notice with pattern 7 in which the total number of resisted antibiotics were only (5 → 8), only Six isolates (P1, P31, P35, P39, P14 and P41) in this study were multi-drug resistant to all antibiotics will use, this high degree of antibiotic resistance may be due to the with widespread usage of broad spectrum antibiotics leading to selective survival advantage of pathogen. Table show blood samples showed variable patterns, while the most isolates from burn multiple resistance centered within the first four patterns while ear, urine and sputum isolated located within same medium group in 4 and 5 patterns. Mah and Toole (2001) noticed the main factor contributing to resistance close cell-cell contact that permits bacteria to more effectively transfer plasmids to one another than in the planktonic state. These plasmids can encode for resistance to several different antimicrobial agents. The biofilm also act as physical protection to bacteria because antimicrobial agents are also ineffective at penetrating the biofilm, decreasing the concentration acting on the bacterial cells within the biofilm and as a consequence their efficacy.

All *Pseudomonas aeruginosa* isolates were subjected to hierarchical cluster analysis. The dendrogram based on dice coefficient values, there were two major clusters when dendrogram was generated on the basis of their antimicrobial sensitivity against 12 antibiotics (Figure 2). The antibiogram patterns of the 60 isolates of *Pseudomonas aeruginosa* of different origin were determined and among them 2 multiple resistance patterns A and B were observed, among A group the isolates clusters in to two sub group.A_1contain 56 isolates while A_2 contain 3 isolates from wound and B group contain only one isolates from wound. The results indicate that all burn isolates were belong to group A, in the same manner all other origin were among group A except *Pseudomonas aeruginosa* wound isolates were within group B. From this comes the importance of this technique to distinguish and differentiate between bacterial strains according to origin of infection beside multi drug resistance pattern, Figure (2) shown 11 real clones were generated and 15 isolates unique patterns. This results indicated that the Appropriate of antibiogram typing as a tool for discrimination isolates and to give idea about different same species isolates from the same patient not always belong to the same clone. results agree with Jacome *et al.* (2012) shown molecular typing for the identification of clonal relationships between isolates of *P. aeruginosa* has been quite frequent in the recent years for simplicity and efficiency also This approach has been considered an important methodology because it allows verifying cross transmission in cases of outbreaks (Janam, 2011).

Table (3): The patterns of antibiotics resistance

Pattern	Total no. of isolates	Site of Infection						Antibiotics resistance	No. of antibiotics resistance
		Burn	Wound	Sputum	Blood	Ear	Urine		
1	12	9	1	-	2	-	-	NOR, CIP TE, AMC, C، CAZ, FOX، TOB, PRL, FEP, CRO, AZM	(10→12)
2	8	5	2	1	-	-	-	TE, AMC, NOR, CIP, C, CAZ, FOX, PRL، FEP, CRO, AZM	(10→11)
3	1	-	-	-	1	-	-	TE, AMC, NOR, CIP, C, CAZ, FOX, TOB، FEP, CRO،	10
4	13	3	1	6	1	1	1	TE, AMC, C, CAZ، FOX, CRO, AZM، TOB, PRL	(7 →9)
5	13	3	4	2	2	2	-	TE, AMC, C, CAZ FOX, FEP, CRO, AZM	(7 →9)
6	1	-	-	-	1	-	-	TE, AMC, C, CAZ، FOX, CRO	6
7	11	-	10	-	-	-	1	TE, C, CAZ, FOX, NOR, CIP, AZM	(5 → 8)

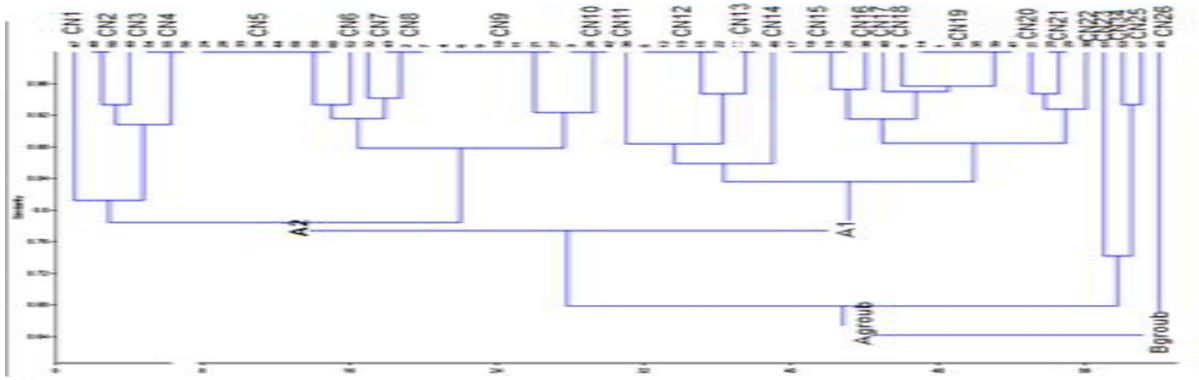

Figure (2): Dendrogram obtained from antibiogram data for *P. aeroginosa*

The ability of *Pseudomonas aeroginosa*. to adhere and form multilayered biofilms on host tissue and other surfaces is one of the important mechanisms by which they are able to persist in the diseases An association is observed between multi-resistance and biofilm production. The biofilm environment seemed to increase genetic exchanges and hence may contribute to multi-resistance phenotypes (Araujo *et al*., 2006).From this point the ability of *pseudomonas aeruginosa* isolates were tested to produce biofilm by two methods; (i.e, Congo red agar method, and Tissue culture plate method).

Congo-red agar method (CRA) This method was described by (Freeman *et al*., 1989) to detect the slime layer production by bacteria using a specially prepared solid medium. The results showed that 68.3% (41/60) isolates produced strong slime layer indicated by formation of black colonies while 31.6% (19/60) isolates were not produced slime layer indicated by formation of pink colonies (Khan *et al*., 2011). This results disagree with Rewatkar and waldher (2010) who indicated that the 90% of isolates gave black colour colonies on Congo red agar plate while only 10% isolates gave pink colour colonies indicating that no biofilm production. as well as These results were in harmony with those reported by Nagaveni *et al*. (2010) who indicated that the 72.7% isolates gave black colour colonies on Congo red agar plate while 27.2% isolates gave pink colour colonies indicating non biofilm.

Tissue Culture Plates Method (TCP) This method that was described by Christensen *et al*., (1985) is most widely used as standard test for the detection of biofilm formation The TCP method was considered to be superior to CRA method. According to (Bose *et al*., 2009) who have shown that the absorbance that are classified into 3 categories (Positive, Moderate and Negative). From the total of 60 clinical isolates, TCP method detected 45% (27/60) as highest value of biofilm formation due to strong adherence 48.3% (29/60) moderate biofilm former and adherence and 6.6% (4/60) as weak or non-biofilm producers. Results observed higher antibiotic resistance in biofilm producing bacteria than non-biofilm producers, for example (p31, P35, P39, P40 and P41) are multidrug resistance and high biofilm producer this results partially agree with Jakribettu *et al*. (2013) who showed 64% of the isolates positive results while 35.9% negative results. The results confirm that the TCP method is a more quantitative and reliable method for the detection of biofilm forming microorganisms as compared to CRA methods, and it can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories (Harvey *et al*., 2007).also used the TCP assay as a simple and rapid method to quantify biofilm formation of different bacterial strains crystal violet is a basic dye known to bind to negatively charged molecules on the cell surface as well as nucleic acids and polysaccharides, and therefore gives an overall measure of the whole biofilm It has been used as a standard technique for rapidly accessing cell attachment and biofilm formation in a range of gram positive and gram-negative bacteria (Matz *et al*., 2005). Biofilm formation provides bacteria with a means of persistently colonizing either living or inert surfaces within a human host (Murray *et al*., 2010).

The hemolysin production by *pseudomonas aeruginosa* was, three types of haemolysins [alpha (α), beta (β) and delta (δ)]. The three haemolysins was tested on the human blood agar and it was found that all these isolates of these bacteria were able to produce hemolysin on blood . *P. aeruginosa* has two pathways to take iron, one of these pathway is hemolysin, and these bacteria produce two hemolysin, it appear to be cytotoxic for most eukaryotic cells, so the hemolysin contribute to invasion through their cytotoxic effects on eukaryotic cells (Gadeberg *et al*., 2000).

Genotypic detection of virulence factor using PCR technology: In this study, two techniques were used for the detection by PCR are multiplex and uniplex. This technique is very sensitive, easy to perform, specific for gene families and very efficient compared with the other methods (Bradford, 2001). Sixty isolates from different hospital in Baghdad used to study five virulence factor (*exoU, exoS, PilB, PlcH, ProteaseIV(TC*)) it was screened by PCR the results show high frequency of virulence factor gene in local isolates. Beginning with the genes codifying for the typeIII secretion system(T3SS) *exoS* and *exoU* were differently distributed among the tested strains, 91.6% (55/ 60) harbored TTSS genes the results show most of the isolates contain either *exoS* or *exoU* neither the both 33(55%) isolates show(*exo U +/exo S*-) while 17 (28.3%) isolates show(*exo U-/exo S* +) 5(8.3%) isolates show (*exo U +/exo S +)* and the last 6 (10%) isolates show (*exo U-/exo S*-) Those that harbour *exoU* gene are referred as cytotoxic that harbour *exoS* are referred to as invasive and those that doesn't harbour any of these genes are considered neither as cytotoxic nor invasive. Therefore, there exists three phenotypes of *P. aeruginosa*, cytotoxic, invasive and neither cytotoxic nor invasive(Choy *et al*., 2008).

Table (4): Prevalence of biofilm formation in *P. aeroginosa*

Biofilm formation	CR-method		TCP-method	
	NO	%	NO	%
High producer	41	68.33	27	45
Moderate producer	-	-	29	48.33
Not producer	19	31.6	4	6.66

Table (5): The percentage of isolates positive for presence of screened virulence genes

Sources	ExoU	ExoS	PilB	TC	PlcH
Burn	18.3%	16.6%	13.3%	20%	-
Wound	25%	1.6%	6.6%	10%	-
Sputum	6.6%	10%	1.6%	10%	-
Blood	8.3%	3.3%	-	3.3%	-
UTI	1.6%	1.6%	-	-	-
Ear swab	3.3%	3.3%	-	3.3%	-

For *pilB* gene, the results showed that 21.6%(13/60) of multidrug resistance isolates of *Pseudomonas aeruginosa* harbour this gene, and the majority of these isolates from male patients .according to infection site (source of isolates) the results showed a widespread dissemination of this gene in *P. aeruginosa* isolated from burn infection 61.5%(8/13) followed by wound infections 30.7%(4/13) and one isolates 7.6%(1/13) from sputum this result partily agree with(Raoof, 2011) who showed that (35%) isolates of *P. aeruginosa* harbour this gene, and widespread dissemination of this gene in *P. aeruginosa* isolated from wound infections (60%), (40%) of ear isolates, then (20%) for each of urine and burn isolates. In the last *proteaseIV (TC)* gene the results showed that 46.6%(28/60) isolates of *P. aeruginosa* harbour this gene, the majority of these isolates from multidrug resistance and male patients widespread of this gene in *P. aeruginosa* isolated from surgical wound and burn patients 64.2% (18/28) because it contribute with tissue injuries fallowed by sputum 21.4% (6/28) this results agree with (Holban *et al*., 2013) who showed most of surgical wound isolates harbour this gene.

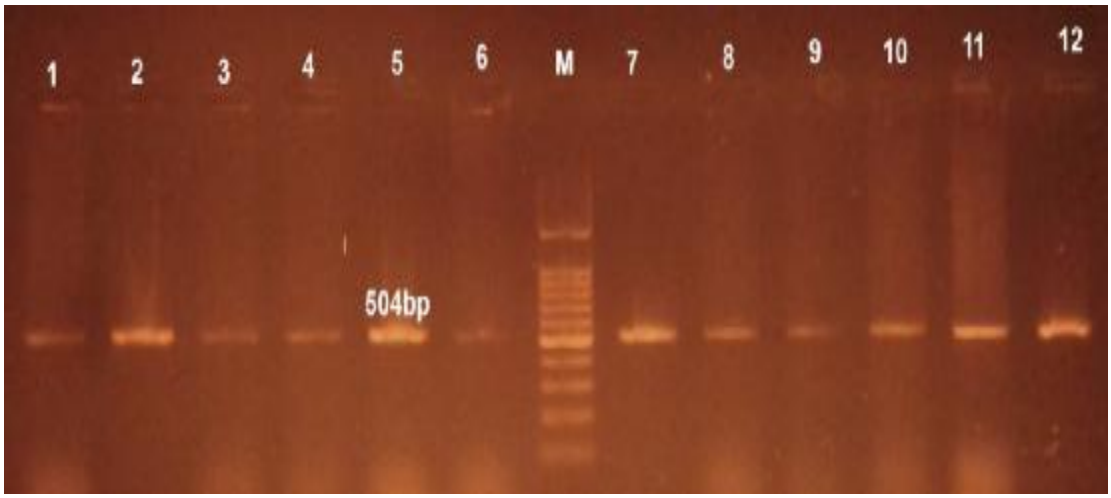

Figure (3): Agaros gel electrophoresis (1% agarose, 7 v/cm2) and ethidium bromide staining to detect exoS gene size product (band bp) 504bp using template DNA prepared by boiling method. (100 bp DNA Ladder); lanes1-12showed positive PCR bands.

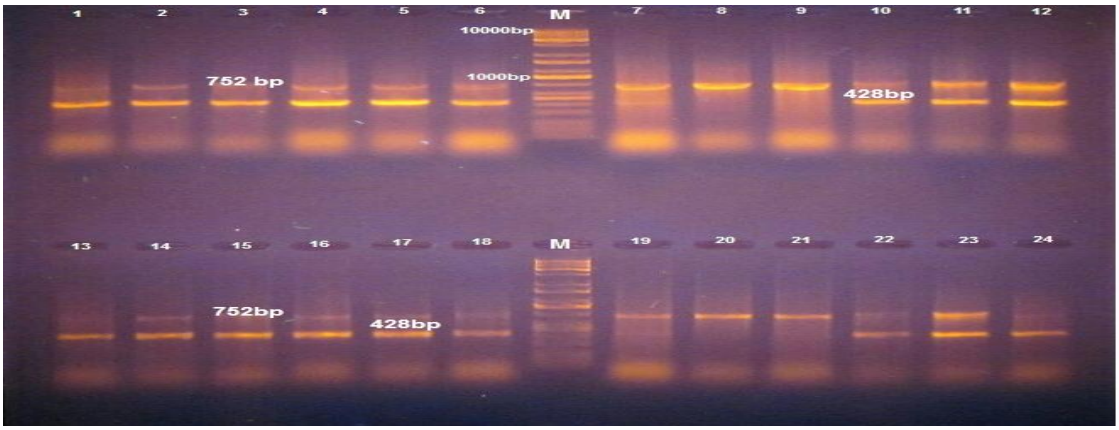

Figure (4): Multiplex PCR: Agarose gel electrophoresis (1% agarose, 7 v/cm2) and ethidium bromide staining. to detect(exoU, proteaseIV) genes size products (bands 428bp, 752bp) respectively using template DNA prepared by boiling method. (100bp DNA Ladder); lanes 1-24 represent of bands.

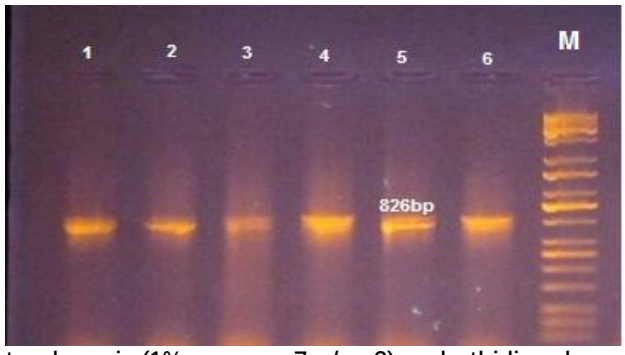

Figure (5): Agaros gel electrophoresis (1% agarose, 7 v/cm2) and ethidium bromide staining to detect PilB gene size product (band bp) 826bp using template DNA prepared by boiling method. (100bp DNA Ladder); lanes1-6showed positive PCR bands.

Conclusions

Detection by housekeeping gene very simple and rapid technique compare with other conventional method, also the antibiogram typing observed as appropriate discrimination isolates and gave idea about different same species isolated from same patients not always belong to the same clone. As well as TCP method very effective to detect the biofilm compare with CRA-method and the last detection of virulence factor by PCR showed most of the *p. aeruginosa* cytotoxic nor invasive and *p. aeruginosa* is able to accumulate several virulence factor associated with multidrug resistance.

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